Original Article

Synchronous Estimation of Glycopyrrolate and Formoterol in Bulk and Pharmaceutical Dosage Form by RP-HPLC Method

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ABSTRACT

Objective: The objective is to develop and validate a fundamental, specific, novel and exact RP-HPLC (Reverse phase-High performance Liquid chromatography) methodology for the synchronous determination of Glycopyrrolate and Formoterol in pharmaceutical dosage form. **Methods and Materials:** The column utilized was BDS C_{18} (250mm x 4.6 mm, 5μm) in isocratic mode, with mobile phase consist of phosphate buffer and acetonitrile (40:60 v/v). The buffer was made by adding 1 ml of Orthophosphoric acid in a 1000ml of volumetric flask and about 900ml of milli-Q water, degas to sonicate and lastly make up the quantity with water. The flow rate used was 1.0ml/ min and effluents were monitored at 241 nm. **Results:** The retention times of Glycopyrrolate and Formoterol are 2.290 min and 2.853 min, respectively. The linearity for Glycopyrrolate and Formoterol are 2.25-13.5μg/ml and 1.2-7.2μg/ml respectively. The recoveries of Glycopyrrolate and Formoterol were observed to be 99.17 to 100.66% and 98.36 to 100.79% respectively. **Conclusion:** The validation was performed for the proposed method and applied successfully for the determination of Glycopyrrolate and Formoterol. The method was found to be precise, accurate for the synchronous determination of Glycopyrrolate and Formoterol in pharmaceutical dosage form.

Key words: Glycopyrrolate, Formoterol, Validation, HPLC.

INTRODUCTION

Glycopyrrolate is a quaternary ammonium salt. Chemically, Glycopyrrolate is (RS)-[3(SR)-Hydroxy-1, 1-dimethylpyrrolidinium bromide] α-cyclopentylmandelate. The chemical formula is $C_{19}H_{28}BrNO_3$. The molecular weight is 398.33g/mol.[1] Glycopyrrolate is a crystalline white powder. It is dissolvable in water and alcohol, and much insoluble in chloroform and ether.[2]

Glycopyrrolate, as other anticholinergic (antimuscarinic) drugs, impedes the action of acetylcholine on structures innervated by postganglionic cholinergic nerves and on smooth muscles that respond to acetylcholine yet require cholinergic innervation. Thus, it diminishes the volume and free acidity of gastric secretions and controls excessive pharyngeal, tracheal, and bronchial secretions.[3]

Formoterol acts as a bronchodilator. It extends the airways of the lungs, so that it helps to inhale all the more effortlessly. It may even be utilized to forestall respiratory issues caused by exercise. It can also be utilized for long-term treatment of chronic obstructive pulmonary disease (COPD).[4]

Chemically, Formoterol is N-[2-Hydroxy-5-[(1RS)-1-hydroxy-2-[[(1RS)-2(4-methoxyphenyl) -1-methylethyl]-amino] ethyl] phenyl] formamide (E)-2-butenedioate dihydrate. The chemical formula is $C_{19}H_{24}N_2O_4.C_4H_4O_4.2H_2O$. The molecular weight is 840.91g/mol.[1]

There are various analytical methods reported in the literature for the assay of

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Glycopyrrolate and Formoterol separately and also with other drugs include spectrophotometry, HPLC, HPTLC and other types.

For only Glycopyrrolate separate RP HPLC methods were available in bulk, tablet dosage forms,[5] and for parenterals.[3] There is also a method for glycopyrrolate alone in human plasma by liquid chromatography–electrospray ionization mass spectrometry[6] and liquid chromatography-Tandem mass spectrometry method for quantification of Glycopyrrolate in horse plasma.[7]

There are various methods for determination for single formoterol alone, like Automated and sensitive method for the determination of formoterol in human plasma by high-performance liquid chromatography and electrochemical detection.[8] There are various RP-HPLC methods for the Simultaneous estimation of Formoterol Fumarate and Tiotropium Bromide,[9] Formoterol Fumarate and Budesonide in metered dose inhaler formulation,[10] spectroscopic method for the simultaneous estimation of Mometasone Furoate and Formoterol Fumarate in Rotacaps.[11] chromatographic methods for the simultaneous determination of Mometasone furoate and Formoterol fumarate dihydrate in a combined dosage form[12] and for Estimation of Formoterol Fumarate and Mometasone Furoate in Metered Dose Inhalation Form by High Performance Liquid Chromatography,[13] UV spectroscopie method for the determination of beclomethasone dipropionate and formoterol fumarate in rotacap dosage form,[4] Simultaneous spectroscopic determination of formoterol fumarate and budesonide in their combined dosage form,[14] RP-HPLC method for estimation of formoterol fumarate and budesonide in pressurised meter dose inhaler form,[15] Simultaneous Reversed-Phase HPLC Method for Formoterol Fumarate and Fluticasone Propionate in Metered dose inhaler.[16]

As per the literature survey no reported method was available for the simultaneous determination of Glycopyrrrolate and Formoterol. The present method was to build up a straightforward, minimal effort RP-HPLC technique for concurrent estimation of Glycopyrrolate and Formoterol in bulk and also in other dosage forms. The method was validated according to ICH guidelines.[17]

MATERIALS AND METHODS

Reagents

Glycopyrrolate and Formoterol were supplied by Astra Zeneca. Acetonitrile, water (HPLC review, Merck) and the various reagents were acquired from M R Enterprisers. The tablets BEVESPI AEROSPHERE (AstraZeneca) containing 8mg of Glycopyrrolate and 25mg of Formoterol were used.

Instrumentation

The LC system comprised of a Waters model 515, PDA detector 2998 with twenty µL sample loop. The output signals were observed and integrated utilizing Empower 2 software.

Chromatographic conditions

The elution method was isocratic. The mobile phase is comprised of a mixture of buffer (1ml of Orthophosphoric acid kept in a 1000ml of volumetric flask, around 900ml of milli-Q water was added, degas to sonicate lastly make up the volume with water) and acetonitrile (40:60 v/v). The mobile phase was filtered through a 0.45-µm (HVLP, Germany) membrane filter before using the same. A BDS C_{18} (250mm x 4.6 mm, 5mm) column was utilized for determination. The rate of flow was 1.0 ml/min and also the column was operated at temperature ~30°C. The sample volume injected was 10µL. The column was equilibrated for not less than 30min with mobile phase flowing through the system prior to injection of the solutions. The wavelength of the UV detector was set at 241nm. A typical RP-HPLC chromatogram of Glycopyrrolate and Formoterol is presented in Figure 1.

Diluent

Water and ACN (50:50).

Standard Preparation

The weighing was done accurately and transferred 9mg of Glycopyrrolate and 4.8mg of Formoterol working Standards into a 100 ml of volumetric flask, added 70ml of diluent, sonicated for 30 min and make up to the final volume with diluent. From the above stock solution, 1ml was taken out in to a 10ml volumetric flask and then make up to the final volume with diluents, to get a concentration of 9mg/ml of Glycopyrrolate and 4.8mg/ml of Formoterol.

Sample Preparation

Around 20 tablets were weighed accurately and crushed to a fine Powder and drug equivalent to 9mg and 4.8mg were kept in a 100ml volumetric flask, dissolved in diluent.

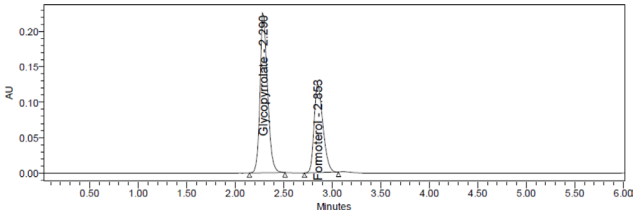

Figure 1: HPLC chromatogram of Glycopyrrolate and Formoterol.

Then 1ml of the above solution was kept into a 10ml volumetric flask and filtered through 0.45µ membrane filter to get concentration of 9µg/ml and 4.8µg/ml for Glycopyrrolate and Formoterol.

METHOD VALIDATION

The developed method was validated as per ICH guidelines[17] for its linearity, accuracy, precision, robustness, and specificity, limit of detection and limit of quantification by using the below mentioned procedures. The validated parameters are provided in Table 1.

System suitability

The validation of system suitability and chromatographic parameters was performed such as tailing factor, asymmetry factor, and number of theoretical plates were calculated.

Linearity

The linearity of this methodology was assessed by linear regression analysis and computed by least square method and studied by preparing standard solutions of Glycopyrrolate and Formoterol at various levels of concentrations. Peak area of the ensuing solutions was determined and additionally the calibration curve was plotted between Peak area versus concentration of the drug in Figure 2 and Figure 3. The response was observed to be linear in the range 2.25-13.5µg/ml and 1.2-7.2µg/ml for Glycopyrrolate and Formoterol. The information was provided in Table 2.1.

Accuracy

Accuracy was demonstrated in triplicate for numerous concentrations of Glycopyrrolate and Formoterol equivalent to 50%, 100% and 150% of the standard quantity were injected into the HPLC system per the test strategy. The typical recovery (%) was computed. The data was given in Table 2.2.

Precision

Precision Repeatability

System precision

Six standard solutions of the similar concentration (100%) were arranged and injected into the HPLC system as per test methodology. The outcomes were given in Table 3.1 (A).

Method precision

Six sample solutions of the identical concentration (100%) were arranged and injected into the HPLC system as per test strategy. The results were given in Table 3.1(B).

Intermediate Precision

Day to Day variability

The study was conducted for two days according to test strategy. The six sample solutions of the identical concentration (100%) were prepared and injected into the HPLC system as per test methodology for Day-1 and Day-2. The results were given in Table 3.2(A).

Instrument to Instrument variability

Two instruments were utilized according to the test method for the study. For Instrument-1 and Instrument-2, six sample solutions of the similar concentration (100%) were arranegd and injected into the HPLC system as per test technique. The results were given in Table 3.2(B).

Table 1: System Suitability Parameters.

Validation parameter (Units)	Results	
	Glycopyrrolate	Formoterol
Linearity range (µg/ml)	2.25 – 13.5	1.2 – 7.2
Regression equation	y = 13136x + 1558.6	y = 16066x + 666.5
Correlation Coefficient(r)	0.999	0.999
System Precision (%RSD)	NMT 0.32	NMT 0.43
Method Precision (%RSD)	NMT 1.03	NMT 0.28
Intermediate Precision (day-day) (%RSD)	NMT 0.25	NMT 0.26
Accuracy	98.36% to 100.66%	98.36% to 100.79%
Robustness (%RSD)		
Flow rate: (0.9ml/min and 1.1ml/min)	NMT 0.28	NMT 1.12
Mobile phase: Buffer : ACN(40:60)	NMT 0.58	NMT 0.98
Assay	99.46 ± 0.61	98.74 ± 0.36

RSD-Relative standard deviation, NMT-Not more than. ACN-Acetonitrile

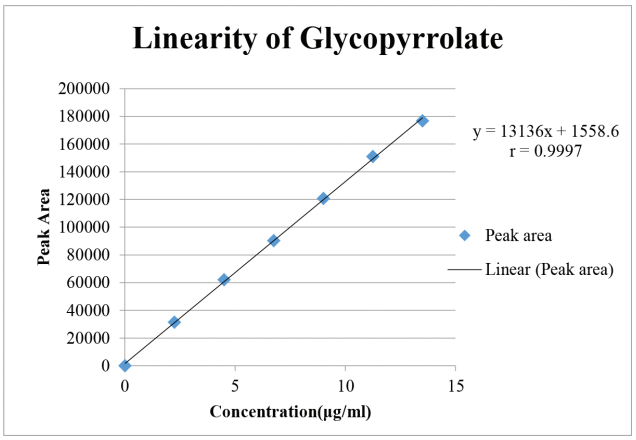

Figure 2: Linearity of Glycopyrrolate in the range 2.25 to 13.5µg/ml.

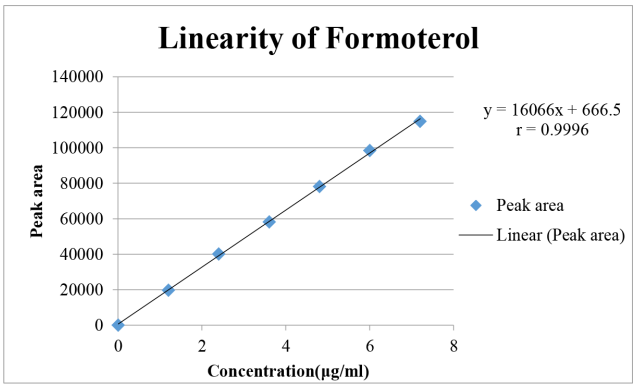

Figure 3: Linearity of Formoterol in the range 1.2 to 7.2µg/ml.

Table 2.1: Linearity Data of Glycopyrrolate and Formoterol.

S.No	Glycopyrrolate			Formoterol		
	Conc(µg/ml)	Rt(min)	Area	Conc(µg/ml)	Rt(min)	Area
1	2.25	2.289	31371	1.2	2.85	19798
2	4.5	2.287	61955	2.4	2.85	40114
3	6.75	2.288	90106	3.6	2.851	58195
4	9	2.286	120649	4.8	2.846	78179
5	11.25	2.287	150911	6	2.847	98380
6	13.5	2.284	176601	7.2	2.847	114863
	r = 0.9997 y = 13136x + 1558.9			r = 0.9996 y = 16066x + 666.5		

Rt-Retention time.

Table 2.2: Accuracy Data of Glycopyrrolate and Formoterol.

S.No		Glycopyrrolate			Formoterol		
	Spiked level	Amount added (µg/ml)	Amount present (µg/ml)	Average %Recovery* + %RSD	Amount added (µg/ml)	Amount present (µg/ml)	Average %Recovery* + %RSD
1(n=6)	50%	2.25	2.23	99.14+ 0.45	1.20	1.21	99.90 + 0.79
2(n=6)	100%	4.50	4.46	99.07 + 0.64	2.40	2.40	99.86 + 0.88
3(n=6)	150%	6.75	6.75	99.99 + 0.75	3.60	3.58	99.38 + 0.90

RSD-Relative standard deviation. *n=6 (Average of 6 determinations)

Table 3.1(A): Precision (Repeatability) – System Precision Data of Glycopyrrolate and Formoterol.

S.No	Glycopyrrolate			Formoterol		
	Conc(µg/ml)	Rt(min)	Area	Conc(µg/ml)	Rt(min)	Area
1	9	2.281	121357	4.8	2.84	79815
2	9	2.284	121377	4.8	2.846	79621
3	9	2.286	122137	4.8	2.848	79104
4	9	2.286	121585	4.8	2.848	79163
5	9	2.288	122276	4.8	2.85	79872
6	9	2.289	121589	4.8	2.852	79801
Mean			121720			79563
SD			392			343
%RSD			0.32			0.43

RSD-Relative standard deviation. Rt-Retention Time.

Table 3.1(B): Precision (Repeatability) – Method Precision Data of Glycopyrrolate and Formoterol.						
S.No	Glycopyrrolate			Formoterol		
	Conc(µg/ml)	Rt(min)	Area	Conc(µg/ml)	Rt(min)	Area
1	9	2.283	123493	4.8	2.85	79864
2	9	2.284	123142	4.8	2.851	79534
3	9	2.285	123209	4.8	2.853	79347
4	9	2.29	122813	4.8	2.853	79644
5	9	2.29	120345	4.8	2.853	79737
6	9	2.291	121332	4.8	2.853	79974
Mean			122389			79683
SD			1260			227
%RSD			1.03			0.28

RSD-Relative standard deviation. Rt-Retention Time.

Table 3.2(A): Intermediate Precision Data (Day To Day Variability) of Glycopyrrolate and Formoterol.				
S.No	Inter-day Precison			
	Day-1		Day-2	
	Peak area		Peak area	
	Glycopyrrolate (9 µg/ml)	Formoterol (4.8 µg/ml)	Glycopyrrolate (9 µg/ml)	Formoterol (4.8 µg/ml)
1	124148	76129	124224	76024
2	124616	76322	124648	76125
3	124723	76509	124337	75946
4	124098	76237	124089	76246
5	124042	75963	124145	75832
6	124027	75982	124702	76366
Mean	124276	76190	124358	76090
SD	310	210	260	197
%RSD	0.25	0.28	0.21	0.26

RSD-Relative standard deviation.

Table 3.2(B): Intermediate Precision Data (Instrument To Instrument Variability) of Glycopyrrolate and Formoterol.				
S.No	Inter-Instrument Precison			
	Instrument-1		Instrument-2	
	Peak area		Peak area	
	Glycopyrrolate (9 µg/ml)	Formoterol (4.8 µg/ml)	Glycopyrrolate (9 µg/ml)	Formoterol (4.8 µg/ml)
1	124454	76957	124422	76428
2	124125	76223	124854	76521
3	124327	76905	124733	75649
4	124980	76373	124982	76523
5	124420	75658	124584	75238
6	124284	75587	124208	76668
Mean	124432	76284	124631	76171
SD	293	588	286	584
%RSD	0.24	0.77	0.23	0.77

RSD-Relative standard deviation. Rt-Retention Time.

Limit of detection and Limit of Quantification

The values of the LOD and LOQ were computed from the calibration curve with the help of slope and standard deviation as per ICH guidance. The LOD and LOQ of Glycopyrrolate were observed to be 0.098µg/ml and 0.298µg/ml respectively. The LOD and LOQ of Formoterol were found to be 0.070µg/ml and 0.213µg/ml respectively. The outcomes were given in Table 4.

Robustness

Robustness was done by little considerable changes within the chromatographic conditions and retention time of Glycopyrrolate and Formoterol were noted. The factors chosen are rate of flow and variation within the composition of mobile phase. The outcomes were not affected by little variations in these parameters as appeared in Table 5(A) and 5(B).

Assay

The assay and purity were computed for brand BEVESPI AEROSPHERE (AstraZeneca) with label claim 9mg and 4.8mg. The observed value was evaluated by comparing with the standard value without impedance from the excipients utilized in the tablet dosage form. The outcomes were given in Table 6.

Degradation studies

Acid Degradation Studies

One ml of 2N Hydrochloric acid was included to 1 ml stock solution of Glycopyrrolate and Formoterol and refluxed for 30min at 600. The resultant solution was diluted to get 9µg/ml and 4.8µg/ml solution and 10 µl solutions were injected into the system and the chromatograms were recorded to evaluate the stability of sample.

Table 4: LOD and LOQ of Glycopyrrolate and Formoterol.

	Glycopyrrolate	Formoterol
LOD	0.098	0.070
LOQ	0.298	0.213

Table 5(A): Robustness Data Relating to Change in Flow Rate (1.0 ml/min).

S.No		Glycopyrrolate			Formoterol		
	Flow rate (ml/min)	Average Peak Area*	SD	%RSD	Average Peak Area*	SD	%RSD
1	0.9ml/min	124674	347	0.28	76497	359	0.47
2	1.0ml/min	124447	200	0.16	76374	380	0.50
3	1.1ml/min	124492	240	0.19	76150	853	1.12

RSD-Relative standard deviation. *n=3 (Average of 3 determinations)

Table 5(B): Robustness Data Relating to Change in Mobile Phase Composition.

S.No		Glycopyrrolate			Formoterol		
	Mobile phase variation (%)	Average peak area*	SD	%RSD	Average peak area*	SD	%RSD
1	M.P-1-(BUFFER:ACN::41:59)	124777	298	0.24	75673	592	0.78
2	M.P-2-(BUFFER:ACN::40:60)	124822	718	0.58	76397	185	0.24
3	M.P-3-(BUFFER:ACN::39:61)	124659	458	0.37	76199	749	0.98

RSD-Relative standard deviation. *n=3 (Average of 3 determinations)

Table 6: Results of Analysis of Laboratory Samples (Assay).

S.No			Glycopyrrolate		Formoterol	
	Sample	Label	Amount found	%Purity + RSD*	Amount found	%Purity + RSD*
1	Brand-1 BEVESPI AEROSPHERE	9mg/4.8mg	8.99	99.46 + 0.61	4.46	98.74 + 0.36

RSD-Relative standard deviation. *n=3 (Average of 3 determinations)

Table 7: Results of Degradation Studies.				
	Glycopyrrolate		Formoterol	
	%Assay	Degraded	%Assay	Degraded
Acid	94.50	-5.50	95.56	-4.44
Alkaline	96.89	-3.11	97.65	-2.35
Peroxide	97.97	-2.03	98.52	-1.48
Thermal	98.63	-1.37	99.39	-0.61
UV	98.52	-1.48	99.70	-0.30
Water	98.86	-1.14	99.28	-0.72

Alkali Degradation Studies

To 1ml stock solution of Glycopyrrolate and Formoterol, 1ml of 2N sodium hydroxide was included and refluxed for 30min at 60^0. The resultant solution was diluted to obtain 9µg/ml and 4.8µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to evaluate the stability of sample.

Oxidation

To 1ml of stock solution of Glycopyrrolate and formoterol 1ml of 20% hydrogen peroxide (H_2O_2) was included separately. The solutions were maintained at 60^0 for about 30 min. The resultant solution was diluted to acquire 9µg/ml and 4.8µg/ml and 10µl were injected into the system and afterwards chromatograms were recorded to evaluate the stability.

Dry Heat Degradation Studies

The standard drug solution was kept in oven in for 6h at 105^0 to check dry heat degradation. For HPLC study, the resultant solution was diluted to 9µg/ml and 4.8µg/ml solution and 10µl were injected into the system and the chromatograms were recorded to evaluate the sample stability.

Photo Stability studies

The drug's photochemical stability was also evaluated by exposing the 9µg/ml and 4.8µg/ml solution to UV Light by keeping the beaker in UV Chamber for 7days in a photo stability chamber. For HPLC study, the resultant solution was diluted to obtain 9µg/ml and 4.8µg/ml solutions and 10µl were injected into the system and the chromatograms were recorded to evaluate the sample stability.

Neutral Degradation Studies

Stress testing was performed under neutral conditions at a temperature of 60º by refluxing the drug in water for 6h. For HPLC study, the resultant solution was diluted to 9µg/ml and 4.8µg/ml solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of the sample.

The results of all the degradation studies were presented in Table 7.

RESULTS

A reverse-phase column methodology was proposed as an appropriate strategy for the concurrent estimation of Glycopyrrolate and Formoterol dosage form. The optimization in chromatographic conditions was performed by changing the composition in mobile phase. The optimization of mobile phase was performed by testing at different ratios. But, buffer and acetonitrile in the ratio 40:60v/v was utilized as mobile phase, which demonstrated good resolution of Glycopyrrolate and Formoterol peak. The wavelength chosen for detection was 241nm, because the drug showed best peak area. The retention times of Glycopyrrolate and Formoterol were 2.290min and 2.853min respectively without intereference from any impurities in the assay. The retention times of Glycopyrrolate and Formoterol are 2.290 min and 2.853 min, respectively. The linearity for Glycopyrrolate and Formoterol are 2.25-13.5µg/ml and 1.2-7.2µg/ml respectively. The recoveries of Glycopyrrolate and Formoterol were observed to be 99.17 to 100.66% and 98.36 to 100.79% respectively.

DISCUSSION

The objective is to develop and validate a fundamental, specific, novel and exact RP-HPLC (Reverse phase-High performance Liquid chromatography) methodology for the synchronous determination of Glycopyrrolate and Formoterol in pharmaceutical dosage form. The same has been developed and this method is simple, economical, rapid, precise accurate and sensitive, as per the information on the drug recovery data and statistical analysis of data. This method can be easily adopted for regular quality control analysis. The outcomes of this analysis affirmed that the method is appropriate for determination of drug in various pharmaceutical formulations without any interference of excipients. Thus the proposed strategy

can be applied in simultaneous estimation of Glycopyrrolate and Formoterol in marketed formulations.

CONCLUSION

This method is fast, precise and sensitive. It makes utilization of less amounts of solvents and changes to the set of conditions can be done within short period of time. This strategy can be suitably applied for the normal examination of Glycopyrrolate and Formoterol in various dosage forms. It doesn't experience the interference because of availability of regular excipients in different dosage forms and can be helpfully applied for quality control examination.

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CONFLICT OF INTEREST

Author declares no conflict of interest.

ABBREVIATIONS

Rt: Retention Time**; RSD:** Relative Standard Deviation; **Concn:** Concentration; **NMT:** Not more than; **ACN:** Acetonitrile.

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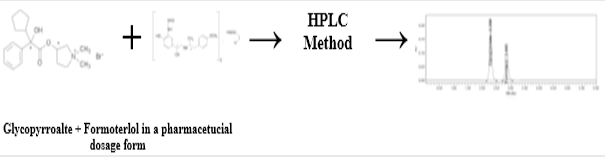
SUMMARY

- Glycopyrrolate, as other anticholinergic (antimuscarinic) drugs, impedes the action of acetylcholine on structures innervated by postganglionic cholinergic nerves and on smooth muscles that respond to acetylcholine yet require cholinergic innervation
- Formoterol acts as a bronchodilator. It extends the airways of the lungs, so that it helps to inhale all the more effortlessly. It may even be utilized to forestall respiratory issues caused by exercise.
- There are various methods for determination for single Glycopyrrolate and Formoterol, and no reported method was available for the simultaneous determination of Glycopyrrrolate and Formoterol.
- The present method was to build up a straightforward, minimal effort RP-HPLC technique for concurrent estimation of Glycopyrrolate and Formoterol and can be applied successfully for bulk and also in other dosage forms.

PICTORIAL ABSTRACT



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